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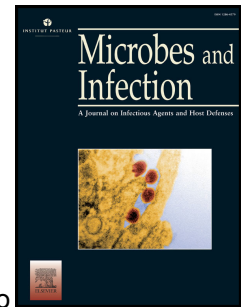
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**Time dependent decline of neutralizing antibody titers in COVID-19 patients from Pune,
India and evidence of reinfection**

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Abstract

Purpose: To assess modulation of neutralizing antibody titers in COVID-19 patients and understand association of variables such as age, presence of comorbidity, BMI and gender with antibody titers.

Methods: Patients (n=100) diagnosed from 20th March 2020-17th August 2020 and treated at two large hospitals from Pune, India were included and followed up (clinical and serologic) for varied periods. IgG-anti-SARS-CoV-2 (Spike protein-based ELISA) and neutralizing antibody titers (NAb, PRNT) were determined in all the samples.

Results: Of the 100 patients enrolled initially (median 60 days of diagnosis), follow up samples were collected from 70 patients (median 106 days of diagnosis). Overall, NAb titers reduced significantly ($p < 0.001$) and as early as 3-4 months. During two visits, 20% and 7.1% patients reported some symptoms. At the first visit, NAb titers were higher in patients with severe disease ($p < 0.001$), comorbidities ($p < 0.005$), age < 50 years ($p < 0.05$) and male gender ($p < 0.05$). Multivariate analysis identified older age ($p < 0.001$), duration post-diagnosis and female gender as independent variables influencing NAb titers (negative correlation, $p < 0.05$). During the follow-up, reduction in NAb titers was recorded in patients with comorbidity ($p < 0.05$), mild disease ($p < 0.05$), age < 50 years ($p < 0.05$), higher BMI ($p < 0.05$) and male gender ($p < 0.001$). Serology identified six cases of asymptomatic reinfections.

Conclusions: Decline of NAb titers was associated with age < 50 years, mild disease, comorbidities, higher BMI and male gender. At the time of follow up, 8/70 (11.4%) patients lacked neutralizing antibodies. Evidence of 6 probable asymptomatic reinfections suggests waning of immunity, but, probable protection from clinical disease needing hospitalization.

Keywords: COVID-19, severity, neutralizing antibodies, comorbidities, BMI, reinfection, decline in titers

Introduction

The unprecedented pandemic of COVID-19 disease caused by SARS-CoV-2 continues to affect global population and economies. So far, the virus has infected 222 countries leading to 212.6 million patients and 4.4 million deaths (<https://www.worldometers.info/coronavirus/#countries>).

To combat the rapid spread of the infection, several vaccines were rapidly developed and made available for immunization of different populations. To assess / predict efficacy of vaccines, it is of utmost importance to understand immunologic basis for recovery from the disease as well as progression to severity. Currently, correlates of protection for COVID-19 are not known. With the emergence of several variants of concern globally, antibody response in general and neutralizing antibody response in particular has gained additional significance.

In view of the decline in antibody titers in other corona viruses [1, 2], understanding of the dynamics of antibody responses in COVID-19 is an important question. Several studies conducted using different ELISAs and virus neutralization / surrogate assays have shown that either the titers remain comparable [3] or decline over time [4-6]. Following the identification of first COVID-19 case in Pune, India on 19th March 2020, we analysed IgG/IgM/IgA antibodies (ELISA) [7], neutralizing antibodies (PRNT) [8] and modulation of circulating immune cells [9] in patients with different clinical presentations examined till ~1month post-disease onset. In patients with severe disease, early and high titers of IgG/IgA and neutralizing antibodies (NAb) were recorded. The next important issue was persistence of the neutralizing antibodies and understanding factors deciding the magnitude and duration of antibody response. In view of the availability of a variety

of vaccines and implementation of national immunization programs, antibody dynamics in natural infection needs to be elucidated in different populations. The present study reports clinical and serological follow up of COVID-19 patients followed for a variable period till ~8 months post-diagnosis.

Material and methods

This study was conducted at two large hospitals, a tertiary care, 840 bedded private hospital and a 300 bedded, Government District General Hospital at Pune, Western India. The enrolled patients were diagnosed from 20th March 2020 to 17th August 2020. For the recruitment of the patients, we first retrieved the list of adult discharged patients from the record sections. The admissions included patients registered for home isolation. Mild, moderate, and severe patients were defined as per clinical guidelines of the Indian Council of Medical Research [10]. The patients who have completed at least one month after diagnosis by RT-PCR (irrespective of the duration of treatment) were contacted telephonically and requested to visit the hospitals for clinical assessment. After obtaining informed written consent on arrival, clinical assessment was done. This included finger SpO₂ after three/six minutes' walk and chest radiography whenever necessary. All the patients who consented for a follow-up visit were included. The samples were collected from 10th August 2020 to 12th March 2021. 2-3ml blood was collected and separated serum samples were stored at -80°C in aliquots.

Serology

All the samples were tested for the (1) presence of IgG-anti-SARS-CoV-2 antibodies by ELISA (SCoV-2 Detect IgG ELISA, Inbios International, Inc., USA) and (2) titers of NAb by plaque

reduction neutralization test (PRNT₅₀) as per the protocol described earlier [8]. The ELISA uses spike protein of SARS-CoV-2 as the coating antigen. As per the manufacturer, this assay is 91.8% specific and 98.9% sensitive. For PRNT, SARS-CoV-2 virus (8004/IND/2020/IRSHA PUNE, accession number MT416726) isolated at IRSHA was used for PRNT using Vero CCL81 cells in 24-well format. Test/control sera were diluted 4-fold, incubated with 20-40 pfu of the virus and on day 5 post-infection, non-neutralized virus was quantitated by plaque counting. PRNT₅₀ titers were calculated using Karber's formula. Samples with PRNT titer less than 10 were considered negative. For statistical analysis, negatives were assigned a titer value of 4.5.

Statistical analysis

For comparisons of PRNT titers, geometric mean titers (GMTs) were used. Multivariate analysis was done to assess the association of various parameters with PRNT titers by calculating the standardized coefficient (Beta). For comparisons, Chi-square test with Yates' correction, unpaired t-test, and ANOVA were used.

Results

Characteristics of the study population

We recruited 100 COVID-19 patients; 81 with mild disease (mean age, 37.8±12.4 years, 46 males and 35 females), 15 with moderate (mean age, 50.6±12.3 years, 11 males and 4 females) and 4 with severe disease (mean age, 53.3±12.2 years, 2 males and 2 females). Overall, fifty-nine males and 41 females were recruited in this study. Twenty-six patients reported at least one co-morbidity (Table1). Proportion of comorbidities was higher in non-mild than mild infections (p<0.001). Except one, all were non-smokers; According to Body Mass Index (BMI), 15 were obese while 29

were overweight (44% with higher BMI). A follow up sample could be collected from 70 patients (59 with mild, 9 with moderate and 2 with severe disease). Of these, 38 were males and 32 were females. Mean age for patients studied at both visits were comparable. The median duration between sample collection and diagnosis of COVID-19 was 60 days (first visit) and 106 days (second visit).

Post-COVID-19 signs and symptoms at both the visits:

Table 1 displays symptoms reported by COVID-19 patients at two time points. At the first visit, 20% (20/100) patients including 3 with comorbidities reported some symptoms. On examination, slight edema on feet, pallor with edema on feet along with puffiness of the face, occasional wheeze, crepitation's, and bilateral wheeze were recorded individually by 5 patients. Hypertension (either systolic ≥ 140 or diastolic ≥ 90) was noticed in 15 patients. Except one, all the 100 patients exhibited SpO₂ saturation of $> 95\%$. This patient was having hypertension with diabetes, was complaining of dyspnea on exertion. Crepitation's were present and chest radiograph showed bilateral localized infiltrations. At follow-up visit, all had SpO₂ saturation of more than 95% except two patients who were not having any complaints. 13/20 patients reporting symptoms at first visit were followed up. Two patients (one with comorbidity) continued to have the symptoms. Overall, 7.1% (5/70) patients examined at second visit reported symptoms (Table 1).

Seropositivity and association of neutralizing antibody titers with the variables examined:

At the time of two visits, 93/100 (93%, first) and 68/70 (97.1%, second) patients circulated IgG antibodies while NAb positivity was recorded in 91/100 (91%) and 62/70 (88.6% patients) respectively. Thus, 2/93 (2.1%) IgG positives at first visit and 6/68 (8.8%) IgG positives at the

second visit lacked neutralizing antibodies. Overall, 11.4% patients were NAb negative at the time of second visit.

Next, we compared NAb titers in the patients screened at both the time points. Figure 1 depicts modulation of NAb titers in relation to the factors considered. Due to small numbers, patients with moderate and severe disease were grouped as non-mild cases for comparing disease severity. At the first visit, higher titers were associated with the presence of comorbidities ($p < 0.005$, figure 1A), non-mild disease ($p < 0.001$, figure 1B), age < 50 years ($p < 0.05$, figure 1C) and male gender ($p < 0.05$, figure 1D). Increased duration between COVID-19 diagnosis and sampling was associated with a significant decline in NAb titers ($p < 0.005$, figure 1F). Multivariate analysis identified age ≥ 50 years ($p < 0.001$), duration between diagnosis and sample collection and female gender (negatively) as independent variables influencing NAb titers ($p < 0.05$ for all).

At the second visit, significant differences were maintained only in relation to disease severity ($p < 0.001$, figure 1B) and older age ($p < 0.05$, figure 1C). During the follow up, reduction in NAb titers was recorded in patients with comorbidity ($p < 0.05$, figure 1A), age ≥ 50 years ($p < 0.05$, figure 1C), higher BMI ($p < 0.05$, figure 1E), male gender ($p < 0.001$, figure 1D) and mild disease ($p < 0.005$, figure 1B).

Persistence of Nabs

During this study, the patients were not recruited during the acute phase. At the time of first sampling, duration between diagnosis and collection varied from 30-179 days (median 60 days) while this duration varied from 60 to 348 days (median 106 days) for second sampling. NAb titers (GMT) reduced from 205 (95%CI - 140 to 300) to 97 (95%CI - 64 to 147, $p < 0.001$). Further, a

separate analysis was carried out to understand modulation of NAbs when sampling was done at different intervals (Figure 2). For 42/70 (60%) patients, the initial sample was collected 30-59 days after diagnosis. For the remaining patients, initial samples were collected at 2-3months (n=9, figure 2B), 3-4months (n=12, figure 2C) and 4-5months (n=5, figure 2D). In addition, 2 patients were sampled for the first time at 5 and 6 months respectively. The first patient was negative for neutralizing antibodies in the initial sample and at 6months follow up. The second patient exhibited a titer of 348 at 6 months that increased to 2560 at 8 months.

Of the 39 patients tested within 1-2 months of diagnosis, a significant decline in NAb titers at 2-3 months (n=19, $p<0.001$) and at 3-4 months (n=20, $p<0.001$) was recorded (figure 2A). Of note, in 2 patients followed up at 7-8 months post-diagnosis, a reduction from 397.5 to 12.4 was recorded while a rise from 1579 to 4042 was noted in one patient examined 8 months post-diagnosis. Among patients initially examined 3-6 months post-diagnosis (figures 2B-2D), a subsequent reduction or similar titers over time were noted. Seroconversion in a patient followed at 6-7 months and rise in titers among patients bled 8 months post-diagnosis are noteworthy. Importantly, the proportions of antibody negative patients increased from 6% (30 to 59 days) to 18.75% (>180 days).

Evidence of reinfection

We identified six possible asymptomatic reinfections during this study (Table 2). All these patients had mild episode at first infection. In one, a clear seroconversion from ELISA/NAb negative to ELISA/NAb seropositivity and a titer of 46 was recorded on 180th day (64 days after first sample collection). In addition, a four-fold rise was seen in 5 patients when the samples were collected 102-299 days post-COVID-19 diagnosis. It is intriguing to note that in two patients with NAbs titers of 107 (day111) and 123 (day58), evidence of reinfection was noted when follow up samples

collected after a gap of 37 and 44 days respectively were tested. Two patients with >2000 NAb titers were infected during the second wave.

Discussion

Though early decline in neutralizing antibodies is well documented in COVID-19, studies on long term persistence in general and neutralizing antibodies in particular are limited. With the emergence of novel mutants that can be partially neutralized by naturally acquired / vaccine-induced antibodies [11, 12], such data gains additional importance. This study is based on the follow up of 70 patients wherein the median duration between sample collections and diagnosis were 60 and 106 days respectively. In the absence of acute-phase samples, we were not able to judge the extent of early decline of NAb titers. However, subsequently a significant decline in NAb titers was evident. Half-life of NAb during the first two months was shown to be lower and doubled later [13].

Initial NAb titers (PRNT₅₀) were influenced by several factors (Figure 1). Multivariate analysis identified age > 50 years ($p < 0.001$), female gender (negatively) and duration between diagnosis and sampling ($p < 0.05$ for all) as independent variables for the induction of higher titers. Of note, age > 50 years was associated with comorbidities that in turn reflected disease severity. In continuation with our earlier observations of high neutralizing antibody titers among patients with severe disease during the acute phase [8], this study revealed that similar differences are maintained during later time points as well. Our results are in line with a cross-sectional study from Japan that included 376 patients examined at 180 (147–224) days post-positive test. Antibody titers were higher in the patients requiring ventilator that was higher than those requiring oxygen

support that in turn was higher than those without such support [3]. Sequential follow up of 39 patients from Japan [4] and 65 patients from UK [14] yielded similar findings. Further, our results confirm association of higher BMI and increased age with elevated titers [15-18].

It is interesting to note that at the time of follow up visit, the difference in NAb titers was maintained only in relation to the older age and severity, severity being higher in the patients ≥ 50 years age. It therefore appears that the rates of antibody decline differ in patient groups categorized on the basis of different parameters. In our study, the decline in antibody titers was associated with comorbidity ($p < 0.05$), age > 50 years ($p < 0.05$), higher BMI ($p < 0.05$), male gender ($p < 0.001$) and mild disease ($p < 0.005$). Thus, except for the patients with severe disease continuing to circulate high NAb titers, these categories with high NAb titers in the initial samples did show a significant decline. At the follow up visit, NAb titers were independent of comorbidity, BMI and gender. In-depth studies are required to understand the basis of differential declines in NAb titers in patient categories with initial high titers.

Of concern, patients with mild disease did exhibit significant reduction in NAb titers. Further, NAb negativity was found only in the mild patients at around 10% (9% at first visit and 11.4% at second visit). Though our follow up is limited and protective levels of NAb are not yet identified, the possibility of reinfection of mild disease patients, especially with divergent strains, cannot be ruled out. Gender-based comparisons have provided contradictory results from no difference [6] to higher titers in males [5] or females [15, 19]. In the previous report [8] as well as this study, males predominated severe disease (80.7% and 68.4% respectively) and may be the reason for observed high titers.

Comparisons of NAb titers among 70 patients from whom a repeat sample was collected at different time intervals led to some useful information (Figure 2). For 60% of these patients, the initial sample was collected 1-2months post-diagnosis. A decline in NAb titers was evident for 39/42 (92.8%) in 2-4months. For patients with longer follow up, the decline was conspicuous. In 2 patients with mild disease tested at 7-8months, the titers were reduced to 12.4 suggesting possibility of subsequent NAb negativity and risk of infection with the emerging viral variants. Interestingly, a 61year old female patient with mild disease exhibited NAb titer of 1579 on day 48 post-diagnosis and 4062 on day 253. Possibility of negative/low titers followed by reinfection cannot be ruled out. A clear seroconversion (NAb and IgG) in one patient tested at 6.5months after a gap of 3 months (Figure 2B) and rise in titers after 8 months (Figure 2C) are noteworthy.

The most significant finding of this study is identification of six cases of reinfection as evidenced by > 4-fold rise in NAb titers. Of note, all these were mild disease patients and hence with lower NAb levels. None of these patients gave history of any symptoms during the follow up period. The observed rise in Nab titers may have been due to probable asymptomatic reinfections in these patients. Importantly, the evidence of reinfection was recorded only in the samples collected 5-10 months post-diagnosis. Of these, two samples collected at 8 and 10 months post-diagnosis showed 14.8fold (from 162 to 2560 after 182 days) and 76.4fold (from 27 to 2090 after 190 days) rise in NAb titers. At this time, Pune was already experiencing second wave of COVID-19. The month of March was predominated by the kappa variant (L452R/E484Q in the Receptor Binding Domain (RBD) of SARS-CoV-2 while from April onwards delta variant was most prevalent [20]. This indicates reinfection of individuals infected during the first wave by the Wuhan-like virus by the two variant viruses causing the second wave. This finding is of special concern in view of the continued emergence of SARS-CoV-2 variants across the globe and observed suboptimal antibody

response of individuals immunized with currently available vaccines [11, 12, 21-23]. An asymptomatic reinfection caused by Pangolin lineage B.1.79 virus was found 142 days after the first episode caused by lineage B.2 virus [24].

The finding of seroconversion at 6.5 months is noteworthy, the previous sample collected on day 116 being negative in PRNT and ELISA. Of concern, for two patients with >100 titer (107 and 123) on days 111 and 58 respectively, evidence of reinfection was noted 37/44 days later. At the time of these infections in October/November 2020, the first wave was ongoing (peak in September) and hence reinfection was most likely caused by the same wild type virus. Rhesus monkeys developing neutralizing antibody titres of ~100 (range 83–197), after primary infection did not show clinical symptoms when challenged 35 days post-primary infection, though replication of the virus was evident [25]. Taken together, though concerns about reinfections caused by variant viruses have been identified during this study, lack of clinical disease is reassuring. These observations suggest role of prior immunity in protection from clinical disease and subsequent progression to severity needing hospitalizations.

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Conflicts of Interest

The authors do not have commercial or other associations that might pose a conflict of interest.

Availability of data

Data used to support the findings of this study are available from the corresponding author upon request.

Author's contributions

PD, JSG, PPD and VAA, conceptualized the study design; PD, MMK, and KKK collected data; PD, JSG, and PPD supervised data collection; PD and KKK clinically assessed the patients; SS

was responsible for serology; VAA supervised laboratory work; VAA and PPD interpreted data; JRP entered and analyzed the data; PPD wrote the manuscript; PD, JSG and VAA critically reviewed the manuscript; all authors approved the manuscript.

Ethics approval

The study started after approval from the institutional ethics committee (DCGI Reg. No. ECR 518/Inst/MH/2014/RR-17) vide REF: BVDUMC/IEC/71, Date: 21/08/2020. Authors obtained informed written consent all the patients for participants and publication.

Legends to the figures:

Figure 1: Depicts neutralizing antibody titers (PRNT₅₀) in the patients studied at first visit (median 60 days post diagnosis) and second visit (median 106 days post-diagnosis). The variables examined include (A) Comorbidity; (B) Disease severity; (C) Age groups; (D) Gender; (E) BMI and (F) Interval between diagnosis and sampling (in days). The data is presented as dot plots with bar representing the geometric mean \pm 95%CI in each group. Each dot represents a single sample. P values were denoted as asterisk. * denotes p-value <0.05, ** denotes p-value <0.01 and *** denotes p-value <0.001. Only significant differences between groups are marked.

Figure 2: Dynamics of neutralizing antibody titers at varied intervals when compared to the initial titers determined at (A) 1-2months post-diagnosis; (B) 2-3months post-diagnosis; (C) 3-4months post-diagnosis and (D) 4-5 months post-diagnosis. The data is presented as dot plots with bar representing the geometric mean in each group.

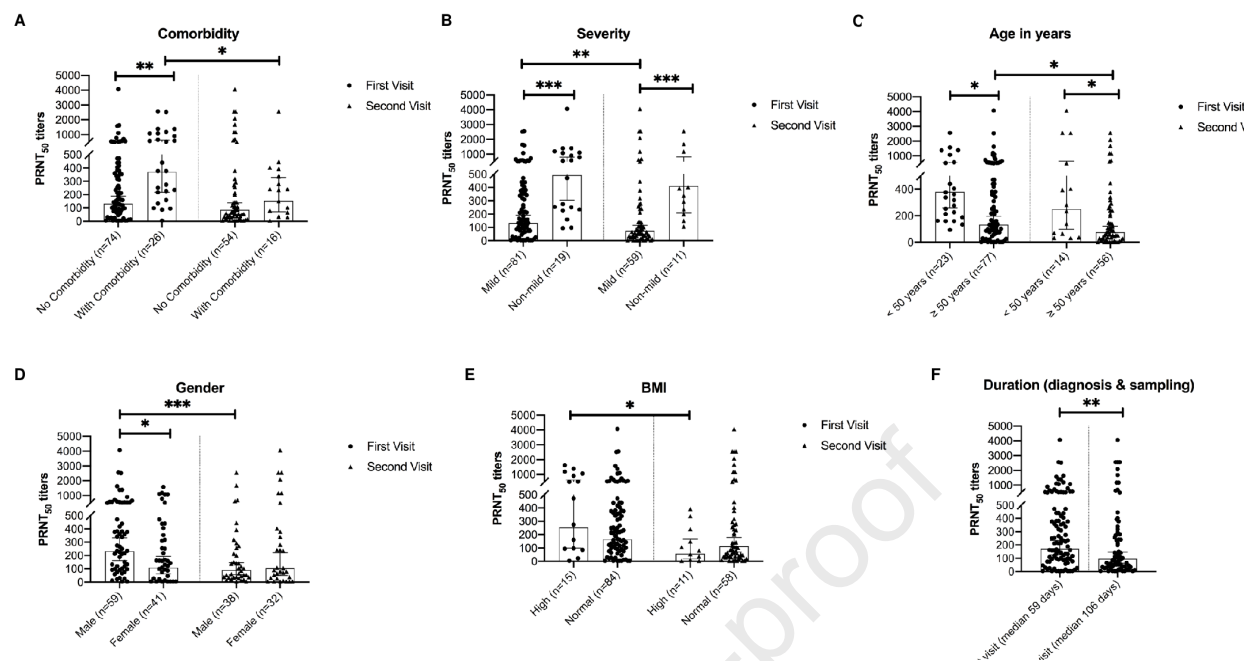
Table 1: Comorbidities and Post-COVID symptoms reported at first and second visits

Comorbidities	No of patients	Symptoms reported	No of patients	
			At first visit (n=100)	At second Visit (n=70)
Hypertension	7	Weakness	5	0
Diabetes	5	Dyspnea on exertion	4	2
Hypertension with diabetes	5	Dry cough	2	0
Hypertension, diabetes and heart disease	2	Fatigue	1	0
Heart disease	1	Intermittent headache	1	0
Hypertension with epileptic seizures	1	Burning micturition	1	0
Chronic renal failure with heart disease and hypertension	1	Burning sensation in the stomach	1	0
Hypertension with hypothyroid	1	White patches in the mouth	1	0
Hypertension with diabetes and hypothyroid	1	Fever with dyspnea on exertion	0	1
Hypertension with vitamin B12 deficiency	1	Mild body ache	0	1
Tuberculosis (under treatment)	1	Frequent chills	0	1
-	-	Dyspnea on exertion with mild cough	1	0
-	-	Dyspnea on exertion with fatigue	1	0
-	-	Joint pains with mild cough	1	0
-	-	Acidity with hemorrhoids	1	0
Total	26/100 (26%)		20/100 (20%)	5/70 (7.1%)

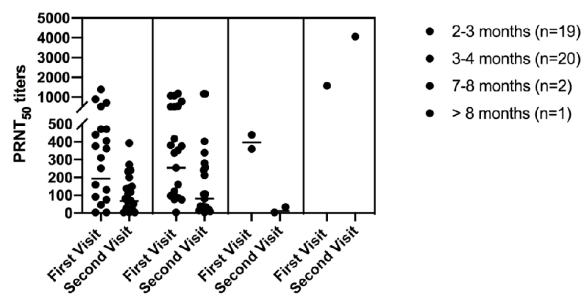
Table 2: Details of the patients showing evidence of reinfection*

Age/Sex	Sample-1 NAb Titer (Days post diagnosis)	Sample-2 NAb Titer (Days post diagnosis)	Duration between the samples (days)	Fold rise in NAb titers
33/M	107 (111)	687 (148)	37	5.4
25/F	13 (126)	90 (206)	80	5.9
26/F	123 (58)	1171 (102)	44	8.5
35/M	Neg (116)	46 (180)	64	seroconversion
50/F	162 (171)	2560 (253)	182	14.8
39/F	27 (109)	2090 (299)	190	76.4

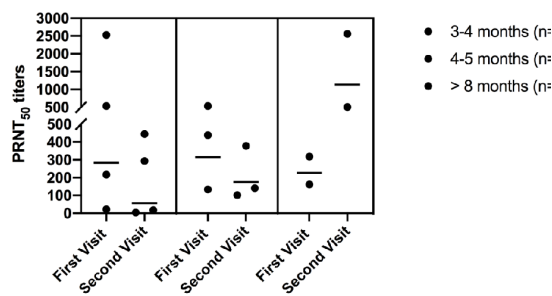
*All had mild disease



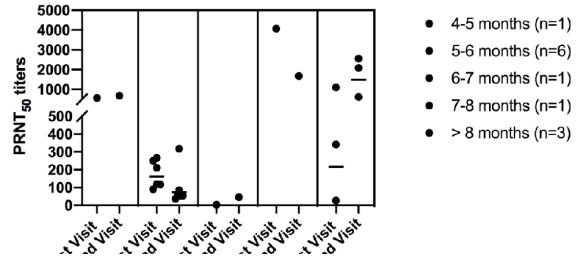
A



B



C



D

